Oral biofilms

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CHAPTER 6

SUMMARY AND CONCLUSIONS

In Chapter 1, a short introduction is given concerning historic developments in oral health care. Although cleaning teeth and the use of toothpastes and mouthrinses has been known for centuries, this was most of the time not intended for the purpose of preserving oral health. The discovery of bacteria in dental plaque by Antonie van Leeuwenhoek in 1676, was the start of the association of bacteria with oral disease. However, it took until 1965 when Löe and coworkers demonstrated in their landmark experimental gingivitis study that bacteria were the cause of gingivitis. Nowadays, the development of toothbrush designs, toothpastes and mouthrinses has become an important industry. Toothbrush designs and the development of powered toothbrushes aim at maximum removal of bacteria, while toothpastes and mouthrinses are developed in order to assist in the control of plaque and caries as well as gingival inflammation, although the exact nature of the synergy between mechanical and chemical plaque control is not always known. Development of new means for mechanical and chemical plaque control can be performed *in vivo*, using groups of volunteers or *in vitro*, using a variety of models each with its own specific property. Although in the end, *in vivo* experiments are inevitable to find out efficacy in the human oral cavity, *in vitro* experiments are the logical first steps in mechanical and chemical plaque control development. In contrast to *in vivo*, *in vitro* experiments are able to give insights into fundamental processes, such as effects of co-adhesion by different strains and species on a substratum. Another advantage is the independence of human volunteers and controllability of *in vitro* experiments. However, models are always a simplification of a complex system. The human oral cavity is inhabited by thousands of different bacterial species, giving rise to extremely complex biofilms. The many possible interactions between bacteria with each other and between bacteria with a substratum surface are complex already on their own, but these interactions

become even more complicated by many other factors, such as the individuals flora, genetic make up of e.g. tissue receptors, immunological responses, salivary composition and diet. Therefore the main aims of this thesis are, (1) to compare different oral biofilm models with respect to their virtues for the evaluation of mechanical and chemical plaque control and, (2) to test the hypothesis that plaque left behind can act as a reservoir for oral antimicrobials, to provide additional substantivity.

Single Strain Oral Biofilm Models as Used for Mechanical Plaque Removal Studies

In Chapter 2, the efficacies of three different modes of contact-brushing on bacterial removal and re-deposition in single strain biofilm models on a saliva-coated surface were compared. *Streptococcus oralis* J22, *Streptococcus mutans* NS or *Actinomyces naeslundii* T14V-J1 were adhered to a salivary pellicle and after 2 h adhesion or 16 h growth, removal and re-deposition were studied. No differences in either removal or re-deposition were observed between the manual, electric rotating or sonic brush. For re-deposition, this suggests that the effect of bacterial footprints is not of influence in single strain experiments. Interestingly, there were significant differences between 2 h adhesion and 16 h growth as well as between the single strains. After 2 h adhesion, removal was on average 93% for *S. mutans* and *S. oralis* and 95% for *A. naeslundii*, while after 16 h growth removal was 94% for *S. oralis* and 86% for *A. naeslundii*. Although the *S. mutans* strain did grow a biofilm, it could not be used in experiments, because of the weak adhesion strength to the surface. Significant differences in bacterial removal as observed between the biofilm models, could be explained by differences in binding strengths to the salivary pellicle surface: the lower the

percentage removal, the stronger the binding strength. The binding strength increased in the following order: *A. naeslundii* T14V-J1 > *S. oralis* J22 > *S. mutans* NS. For 2 h adhesion experiments, after re-deposition the percentage regained is significantly lower for *S. oralis* (75%) than for *S. mutans* or *A. naeslundii*, which both return to around 100%. The lower fractional surface coverage for *S. oralis* J22 after 2 h re-deposition compared to 2 h deposition before brushing, suggests the removal of specific adhesins in the pellicle by brushing. The 16 h old biofilms show a similar result: *S. oralis* regains to 18%, whereas *A. naeslundii* regains to 51%. The lack of differences in either removal or re-deposition between brushing modes, is explained by the fact that brushing was done in a contact mode, usually highly effective in these models. In conclusion, the choice of a given bacterial strain is of great importance in *in vitro* studies on mechanical plaque removal, as different strains of early colonizing bacteria clearly have different binding strengths to the salivary pellicle. In particular, caries associated *S. mutans* was easily removed. The weak binding strength of *S. mutans* NS to salivary pellicles may reflect its characteristic as a late colonizer of dental hard surfaces *in vivo* and therewith its absence in the composition of initial plaque.

Dual-species and Multi-species Oral Biofilm Models as Used for Mechanical Plaque Removal Studies

Single species biofilms lack the interaction between strains as present in multi-species biofilms, such as dental plaque *in vivo*. Therefore the aim of Chapter 3 was to compare dual-species biofilms and multi-species biofilms (human whole saliva) after adhesion or growth, with respect to their ease of removal by different modes of brushing and bacterial re-deposition after brushing. Influence of bacterial footprints

on re-deposition of bacteria was not observed. For the co-adhering dual-species biofilms, we used *A. naeslundii* T14V-J1 and *S. oralis* J22, while fresh human whole saliva was used as a multi-species source for bacterial adhesion and biofilm growth. No differences in either removal or re-deposition were observed between the manual, electric rotating or sonic brush. However, (as in Chapter 2) there were significant differences between 2 h adhesion and 16 h growth as well as between biofilm models. The binding strength, as concluded from the percentage removal revealed opposite effects between the dual-species and multi-species biofilm models. Dual-species biofilms showed significantly stronger binding after growth, in contrast to multi-species biofilms, which showed significantly weaker binding after growth. After re-deposition, the percentage regained was calculated by comparing newly adhering bacteria to the level of bacterial adhesion before brushing. For both dual-species as well as multi-species biofilms, the percentage regained is significantly higher after 2 h re-deposition (53% and 95%, respectively) than after 16 h growth (28% and 7%, respectively). As for Chapter 2, the lack of differences in either removal or re-deposition of bacteria between brushing modes, is explained by the fact that brushing was done in a contact mode. Although our multi-species biofilms closely resemble clinical reality, dual-species biofilms are preferred due to their accurately controllable composition and the weak binding of multi-species biofilms. Moreover, the dual-species biofilms adhere strongly, and are therewith a good "worst case" model for use in mechanical plaque removal studies *in vitro*. In Chapter 4, we compared contact and non-contact removal of single and dual-species (*A. naeslundii* T14V-J1 and *S. oralis* J22) biofilms as well as of multi-species biofilms grown from fresh human whole saliva *in vitro*, using the biofilm models compared in Chapters 2 and 3. Bacteria were adhered to a salivary pellicle for 2 h or grown after adhesion for 16 h

after which their removal was evaluated. In a contact mode, no differences were observed between the manual, electric rotating or sonic brushing and removal was on average 39%, 84% and 95% for *S. mutans* NS, *S. oralis* J22 and *A. naeslundii* T14V-J1, respectively and 90% and 54% for the dual- and multi-species biofilms, respectively. All differences observed in percentage removal between the biofilm models were significant, except for dual-species biofilms compared to *A. naeslundii* as well as for multi-species biofilms compared to *S. mutans*. However, in a non-contact mode, electric rotating and sonic brushes still removed significantly more bacteria (24-40%) than the manual brush as a control (5-11%). Single strain *A. naeslundii* and dual-species biofilms were more difficult to remove after 16 h growth than after 2 h adhesion (on average 62% and 93% for 16 and 2 h old biofilms, respectively), while in contrast biofilms grown from whole saliva were easier to remove (97% and 54% for 16 and 2 h old biofilms, respectively). Considering the strong adhesion of dual-species biofilms, and their easier, more reproducible growth compared with biofilms grown from whole saliva, dual-species biofilms of *A. naeslundii* and *S. oralis* are to be preferred for use in mechanical plaque removal studies *in vitro*.

Antibacterial Activity of Natural Antimicrobials in Toothpaste Formulations Against Oral Biofilms *In Vitro*

With respect to a potential synergy between mechanical and chemical plaque control, it is important to notice that, irrespective of the biofilm model considered and similar to our *in vitro* results, also *in vivo* 100% removal of oral biofilm is impossible and plaque is always left behind (Haps *et al*., 2008). Therefore, we posed the hypothesis that plaque left behind can act as a reservoir for oral antimicrobials, providing

additional substantivity. This hypothesis was addressed in Chapter 5, where we studied the *in vitro* antibacterial efficacies of a herbal- and chitosan-based toothpaste formulation (Parodontax® and Chitodent®) and compared them with a chlorhexidine containing mouthrinse (Corsodyl®), as a positive control and adhesion buffer, as a negative control. Antibacterial efficacy was evaluated by acute bacterial killing, removal and prevention of re-deposition against initial and mature dual- and multi-species oral biofilms. The total biofilm volume of the dual-species mature biofilm amounted 10.9 ± 3.4 $\mu m^3/\mu m^2$, which is about four-fold thicker than an initial dual-species biofilm (2.4 ± 1.0 $\mu m^3/\mu m^2$). Mature multi-species biofilms grown from saliva had a significantly smaller biofilm volume (2.1 ± 0.8 $\mu m^3/\mu m^2$) than dual-species biofilms. Biofilm volumes after initial adhesion of bacteria from whole saliva (multi-species biofilms) were only around 0.2 $\mu m^3/\mu m^2$ and therewith too small for further analyses. The herbal- and chitosan-based toothpastes showed comparable acute killing of oral biofilm bacteria as chlorhexidine. Moreover, treatment of a mature biofilm with a paste or the positive control rinse, yielded ongoing killing of biofilm bacteria even during re-deposition after treatment. This prolonged activity suggests that biofilm may act as a reservoir for oral antimicrobials. Multi-species biofilms had a somewhat larger resilience than dual-species biofilm in achieving re-deposition after treatment, probably due their larger variety of strains and species than present in a dual-species biofilm.

The most striking difference between initial and mature oral biofilms appeared to be that, opposite to our observations on mature biofilms, there was no ongoing decrease in bacterial viability during the re-deposition phase after chemical treatment of an initial biofilm. Initial oral biofilms possess maximally one or two layers of bacteria and evidently have an insufficient sponge capacity to retain antimicrobials in

an amount that enables release in concentrations that are high enough to kill re-depositing bacteria. Thicker, mature biofilms apparently have that ability, which may imply a synergistic mechanism between plaque left behind after brushing and oral antimicrobials. Such a potential synergistic mechanism between (inadequate) mechanical and chemical plaque control is new, and has been further evaluated in an *in vivo* study from our department (Otten *et al*., 2010).

Conclusions

In conclusion, a better insight in the use of biofilm models for mechanical and chemical *in vitro* plaque studies is given in this thesis. When performing mechanical plaque removal studies *in vitro*, the use of 16 h dual-species biofilms (as used in this thesis) is advised. This strongly adhering and thick biofilm is difficult to remove, presenting a model in which differences between brushes are best observed. The performances of electric rotating and sonic brushes are much better than of a manual brush, but never sufficient to remove all biofilm. When chemical plaque control is under investigation, the biofilm model should be able to provide insight into the efficacy of the antimicrobial, as in removal and killing of bacteria and the prevention of re-deposition.

In vitro, natural antimicrobials containing toothpastes Parodontax® and Chitodent® were effective antimicrobial toothpastes, with acute efficacies comparable to chlorhexidine. The effects were most pronounced on thicker 16 h old biofilms and ongoing after treatment and during bacterial re-deposition, suggesting that clinically, antimicrobial oral health care benefits might be gained from antimicrobial absorption in plaque left behind, which is inevitably present in regions where plaque is difficult to remove and biofilm is left after brushing. Especially in patients with orthodontic

appliances or having an otherwise compromised ability to remove plaque mechanically, this might be of additional benefit.

Reference List

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